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The role of salt and shear on the storage and assembly of spider silk proteins

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ABSTRACT

Major ampullate silk fibers of orb web-weaving spiders have impressive mechanical properties due to the fact that the underlying proteins partially fold into helical/amorphous structures, yielding relatively elastic matrices that are toughened by anisotropic nanoparticulate inclusions (formed from stacks of β-sheets of the same proteins). *In vivo* the transition from soluble protein to solid fibers involves a combination of chemical and mechanical stimuli (such as ion exchange, extraction of water and shear forces). Here we elucidate the effects of such stimuli on the *in vitro* aggregation of engineered and recombinantly produced major ampullate silk-like proteins (focusing on structure–function relationships with respect to their primary structures), and discuss their relevance to the storage and assembly of spider silk proteins *in vivo*.

1. Introduction

Orb web-weaving spiders produce a number of task-specific silk protein-based fibers. One of these fibers is assembled from proteins produced by the major ampullate (MA) silk gland. MA silk fibers are used both in the construction of the web as the frame upon which to attach the capture spiral fibers (flagelliform silk fibers coated with aggregate silk) and as a lifeline to escape from predators (Aprhisiart and Vollrath, 1994; Lin et al., 1995). MA silk fibers of orb web-weaving spiders have impressive mechanical properties due to the fact that the underlying proteins partially fold into helical/amorphous structures yielding relatively elastic matrices that are toughened by anisotropic nanoparticulate inclusions (formed from stacks of β-sheets of the same proteins) (Gosline et al., 1999). The primary structures of MA proteins are reminiscent of amphiphilic multiblock copolymers (Exler et al., 2007; Geisler et al., 2008; Zbilut et al., 2005, 2006), in which the repetitive sequence elements (typically composed of multiple repeats of A_n, $(GA)_n$, $(GGX)_n$, and $(GPGXX)_n$) are flanked by highly conserved non-repetitive (NR) amino- and carboxy-terminal domains (Bini et al., 2004; Foo et al., 2006; Hedhammar et al., 2008; Lewis, 2006; Rising et al., 2006).

In common with other silk proteins, the MA proteins of *Araneus diadematus* spiders (ADF-3 and ADF-4) are stored at very high concentrations (>30 wt.%) without the onset of undesirable aggregation within the lumen of the spider. However, our *in vitro* studies of the repetitive sequence elements of recombinantly produced proteins based upon the consensus sequences of the MA proteins of *Araneus diadematus* spiders (Guerette et al., 1996) without the NR terminal domains clearly demonstrate that these proteins are not soluble at such high concentrations as found *in vivo* (Huemmerich et al., 2004a, 2004b). Our findings suggest that the NR terminal domains control both the storage of the proteins (by increasing their solubility and/or deterring undesirable aggregation events) and fiber production (allowing/facilitating controlled protein assembly into fibers upon demand) (Hagn et al., in press).

In vivo Raman spectromicroscopy (Lefevre et al., 2008, 2007b) and *in vitro* ^{13}C NMR (Hijirida et al., 1996) studies of the spinning dope indicate that the repetitive sequence elements adopt highly hydrated random coil and 3_1-helix (polyproline II-like) secondary structures which can be easily transformed into β-sheets. We (Huemmerich et al., 2004b) and subsequently others (Huang et al., 2006; Ittah et al., 2007, 2006; Lin et al., 2009; Stark et al., 2007) have shown the C-terminal NR domains to adopt a predominantly α-helical conformation *in vitro*. These domains have been postulated to form the outer layer of droplet-like structures observed in the spinning dope *in vivo* (Jin and Kaplan, 2003; Lin et al., 2009; Vollrath and Knight, 1999). We have also shown that the C-terminal NR domain is a prerequisite of certain supramolecular self-assembly processes (manifested in the form of fully reversible lower critical solubilisation temperature behavior) of our recombinantly produced proteins (Exler et al., 2007).

In vivo the transition from soluble protein to solid fibers involves a combination of chemical and mechanical stimuli (such as ion exchange, acidification, extraction of water and shear forces (Bini et al., 2004; Foo et al., 2006; Heim et al., 2009; Vollrath and Knight, 2001) that have also been demonstrated to promote protein assembly into fibers *in vitro* (Hardy and Scheibel, 2009a; Hardy et al., 2008; Rammensee et al., 2008). We were consequently

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interested in determining the effect of such stimuli upon the stability of our MA silk-like proteins (Hardy and Scheibel, 2009b).

A protein's solubility in aqueous solution is governed by many factors, of which, one of the most important is its primary structure, which also determines it's secondary, tertiary and quaternary structures (Levy et al., 2006; Rossmann and Argos, 1981; SenGupta and Scheibel, 2007). The presence of other solutes (such as ions) also has an effect upon a protein's solubility in water, as they interfere with the highly ordered layer of water (known as the hydration layer) on the protein's surface (Gerstein and Chothia, 1996; Kim and Cremer, 2001; Lesk et al., 1980; Zhang and Cremer, 2006). Low concentrations of salt tend to improve the solubility of proteins (known as 'salting in'), due to the formation of ion-rich hydration layers in the vicinity of charged and polar amino acid residues (as described by the Debye–Hückel theory), whereas high concentrations of salt tend to have the opposite effect, causing the protein to precipitate (known as 'salting out'). The magnitude of this effect is dependent upon the particular ions and usually follows the Hofmeister series, in which 'chaotropic' ions favor salting-in of proteins and 'kosmotropic' ions favor salting-out of proteins, and anions are well-known to have a much greater effect than cations (Baldwin, 1996; Geisler et al., 2008; Gurau et al., 2004; Horinek et al., 2008; Pegram and Record, 2008; Pirzer et al., 2009; Zhang and Cremer, 2006; Zhang et al., 2005).

Knight and Vollrath have investigated the MA gland of *Nephila edulis* spiders, finding that the physiological concentration of sodium chloride in the spinning dope in the lumen to be of the order of 150 mM, and the total concentration of salts was observed to decrease during the spinning process. They also found that as the dope flowed through the spinning duct, sodium cations were replaced by potassium cations, yet other metal cations (e.g. calcium, copper or magnesium) were almost undetectable in the lumen, duct and the naturally spun fibers. Furthermore, they observed that weakly chaotropic chloride anions are replaced with strongly kosmotropic phosphate anions (significantly increased concentrations) and sulfate anions (small increase with a relatively high level of experimental error) during the passage of the spinning dope along the spinning duct (Knight and Vollrath, 2001). Here we investigate the effects of salts (sodium chloride and sodium phosphate) and shear on the *in vitro* aggregation of our recombinantly produced MA silk-like proteins, and discuss their relevance to the storage and assembly of spider silk proteins *in vivo*.

In this study we use recombinantly produced silk proteins based on the consensus motifs of the MA silk *Aranaeus diadematus* fibroin 3 (ADF3) from the garden cross spider. The repetitive core domains of our proteins are composed of two different sequence modules, denoted A and Q, where module A is a hydrophobic polyalanine-rich motif, and module Q is a more hydrophilic glutamine- and glycine-rich motif (see materials and methods). In this study we use proteins containing 12 or 24 repeats of (AQ). The C-terminal NR3 domain is derived from *adf3* (gi|1263286, obtained from J. Gosline, Vancouver, BC) by using PCR. This domain is mainly α-helical and dimerises via disulfide bond formation between the single cysteine residues contained in two individual NR3 domains (Huemmerich et al., 2004b) (Fig. 1); therefore proteins bearing the NR3 domain are dimeric under non-reducing conditions.

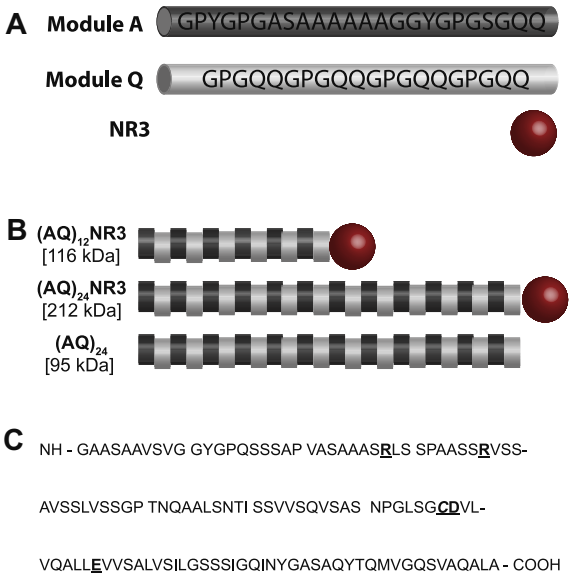

Fig. 1. Employed recombinantly produced proteins based on the consensus sequences of the major ampullate protein (ADF-3) of *Araneus diadematus* spiders. (A) A schematic representation of the repetitive sequence elements (A, hydrophobic polyalanine-rich motif and Q, hydrophilic glutamine- and glycine-rich motif) and the C-terminal non-repetitive domain (NR3). (B) A schematic representation of the engineered and recombinantly produced major ampullate silk-like proteins composed of the elements shown above. For each protein the molecular weight in kDa is given in brackets. Note that proteins bearing the NR domain are dimeric due to interdomain disulfide bond formation. (C) The primary sequence of the C-terminal non-repetitive domain (NR3) of the major ampullate protein (ADF-3) of *Araneus diadematus* spiders, highlighting the residues involved in salt bridges and the cysteine residue responsible for interprotein dimerization. In the $(AQ)_{12}$NR3 (D93A) mutant the single aspartic acid residue is replaced by an alanine residue, inhibiting the formation of one of the two possible salt bridges.

2. Materials and methods

2.1. Cloning, protein expression and purification

The proteins $(AQ)_{12}$NR3 (dimer of 116 kDa), $(AQ)_{24}$NR3 (dimer of 212 kDa) and $(AQ)_{24}$ (monomer of 95 kDa), containing the repetitive elements (A, hydrophobic polyalanine-rich motif: GPYGPGASAAAAAAGGYGPGSGQQ; Q, hydrophilic glutamine- and glycine-rich motif: GPGQQGPGQQGPGQQGPGQQ) were cloned, expressed and purified as previously reported. A D93A mutant (numbering according to the residues of the NR domain; see Fig. 1C) of $(AQ)_{12}$NR3 was obtained according to the QuikChange protocol from Stratagene (USA).

2.2. Salt-induced aggregation assay

Experiments were carried out in accordance with our previously described protocol (Huemmerich et al., 2004b). Prior to the experiment the proteins were dissolved in guanidinium thiocyanate solution (6 M) and then dialyzed against 10 mM Tris/HCl, pH 8.0, 50 mM NaCl. The assay was started by the addition of buffered (10 mM Tris/HCl, pH 8.0) solutions of NaCl and NaH_2PO_4 (pH 8.0). Protein concentrations were, respectively, 8.8 μM, 5.3 μM and 4.7 μM for AQ_{12}NR3 (wt and D93A), AQ_{24} and AQ_{24}NR3. After 1 h of incubation at 25 °C the samples were centrifuged at 17700 g to remove any visible aggregates, and the amount of soluble protein remaining in the supernatant was determined using a UV spectrometer (NanoDrop ND1000, ThermoFischer, USA).

2.3. Shear-induced aggregation assay

Prior to the experiment the proteins were dissolved in guanidinium thiocyanate solution (6 M) and then dialyzed against 10 mM Tris/HCl, pH 8.0. Then the proteins were diluted into buffer containing 10 mM Tris/HCl, pH 8.0 with 50 mM NaCl. Final protein concentrations were adjusted to 17.6 μM of AQ_{12}NR3 and 10.6 μM of AQ_{24}. The samples were incubated for 16 h at 25 °C without/with rotation at 25 rpm (Intellimixer RM-2, NeoLab, Germany). After rotation, the clearly visible aggregates were transferred onto